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ORIGINAL PAPER

Biodiversity of endophytic fungi from seven herbaceous medicinal plants of Malnad region, Western Ghats, southern India

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Abstract: A total of 3611 fungal isolates were recovered from 4200 leaf segments incubated from 7 medicinal herbs during monsoon, winter and summer seasons. These fungal isolates belonged to teleomorphic Ascomycota (23.5%), anamorphic Ascomycota producing conidiomata (17.4%), anamorphic Ascomycota without conidiomata (46.9%), Zygomycota (1.42%) and sterile forms (10.6%). *Chaetomium globosum*, *Aspergillus niger*, *Aureobasidium pullulans*, *Curvularia lunata*, *Fusarium* spp., *Penicillium* spp., *Pestalotiopsis* spp., *Trichoderma viridae*, *Cladosporium cladosporioides*, were frequently isolated from more than one host plant. The number of endophytic isolates was higher in winter than in monsoon and summer seasons.

Keywords: endophytes, diversity, herbs, seasonality, Malnad region

Introduction

Endophytes are the microbes that colonize living internal tissues of plants without causing any immediate overt symptoms (Petrini 1986). They are found in almost all plants studied, including liverworts, hornworts, mosses, lycophytes, equisetopsids, ferns and seed plants from arctic to the most biologically diverse tropical forests (Bacon and White 2000). Investigations of endophytes over the past four decades have increased in number due to characteristics of endophytes including cryptic life style, ubiquitous presence and contributions to host plant survival or vigor (Fisher et al. 1986). The host-fungal symbiosis may be beneficial to hosts in conferring resistance to insects, pathogens and herbivores (White and Cole 1985; Clay 1988; Shankar et al. 2006), reducing physiological costs in terms of water relations, photosynthesis, stress and drought tolerance (Redman et al. 2002; Marquez et al. 2007; Rodrigues et al. 2009), and enhanced vegetative growth (Ernst et al. 2003). Plant-associated microbes have also been recognized for their ecological roles influencing host populations, plant communities (Clay and Hollah 1999; Rudgers and Clay 2007), biosynthesis, biotransformation and biodegradation (Koide et al. 2005; Yu and Dai 2011). Individual plants can harbor dozens of endophytic fungal species (Arnold and Lutzoni 2007) and these endophytes contribute to the hyper diversity of fungi (Hawksworth 2001). Surveys in tropical moist forests suggest that most of the undiscovered endophyte diversity occurs in tropical plants (Frohlich and Hyde 1999; Arnold 2008). Endophytic fungi are repositories of novel metabolites of pharmaceutical importance (Strobel et al. 2004). The ability of endophytes to secrete substances *in vitro* that limit the growth of other microbial species including pathogens has contributed to current enthusiasm for bioprospecting and biological control with endophytic fungi (Gunatilaka 2006; Strobel and Daisy 2003). Many natural products have been isolated from endophytes (Pelaez 2005), including alkaloids, terpenoids, steroids, quinons, isocoumarins, lignans, phenyl propanoids, phenols and lactones (Zhou et al. 2009; Aly et al. 2010). In this context, endophytes might contribute to solving plant, animal and human health problems in response to the increasing threats from drug resistant strains of plant and human pathogens.

Medicinal herbs are an important group of hosts for endophytic fungi (Huang et al. 2008). Endophytes from Chinese medicinal plants show efficacy as pharmaceutical and agricultural compounds (Shentu et al. 2007; Kusari et al. 2008). In this study we documented the diversity of culturable endophytic fungi and their seasonal distribution patterns in herbs of Malnad region, southern India.

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Material and methods

Sample collection and isolation of endophytes

Apparently healthy leaf samples of 7 medicinal plants (Table 1) growing at various sites in the Malnad region were collected, brought in sterile polythene bags to the laboratory, and processed within 24 h of collection. From each host 200 segments were randomly selected from the leaves of two individuals/season that were located within 1 km of each other. Surface sterilization of samples was done by cleaning leaves under running tap water and cutting them into 1 cm segments followed by stepwise washing with 95% ethanol (10 s), 10% chlorine bleach (0.525% Naocl) for 2 min and 70% ethanol for 2 min followed by two rinses in sterile distilled water. Leaf segments were then allowed to surface dry under sterile conditions. This method of surface sterilization has been shown to effectively eliminate contaminants from endophyte cultures (Arnold et al. 2000). Leaf segments were placed on 9 cm Petri plates containing potato dextrose agar medium (PDA, Hi Media Laboratories, Mumbai, India) amended with streptomycin 250 ($mg \cdot L^{-1}$) to suppress growth of bacteria. The efficacy of surface sterilization was confirmed by pressing the sterilized leaf segments on to the surface of PDA medium. The absence of growth of any fungi on the medium confirmed that the surface sterilization procedure was effective (Schulz et al. 1993). Petri plates were incubated at (28±1) ºC with a 12 h photoperiod, and sporulation was induced by incubation in a light chamber under near UV light for 1 to 12 d. Fungi growing out from the leaf segments were subsequently transferred onto fresh PDA plates. Pure cultures were spread on fresh PDA slants. Endophytic fungal species were identified on the basis of cultural characteristics and morphology of fruiting bodies and spores by using standard keys (Barnett and Hunter 1998; Subramanian 1983; Sutton 1980). Cultures that failed to sporulate were recorded as sterile forms. All the isolates were numbered and maintained in Culture Collection Centre of Department of Applied Botany, Kuvempu University, Shankaraghatta, India.

Table 1: Herbaceous plants studied for the isolation of endophytic fungi from Malnad region of Karnataka and their medicinal uses

Host Plant	Family	Collection site	Elevation	Medicinal uses
Achyranthes aspera L.	Amaranthaceae	Shimoga	13°55'N, 75°34'E, 569 m	Demulcent, alterative
Eclipta alba (L.) Hassk	Asteraceae	Kumsi	14°07'N, 75°4'E, 674 m	Asthma, Night blindness
Elephantopus scaber L.	Asteraceae	Shankaraghatta	13°55' N, 75° 34' E, 720 m	Earache, ulcer and eczema
Leucas aspera Spreng.	Lamiaceae	Lakkavalli	13°42N, 75°39'E, 300 m	chronic rheumatism
Ocimum sanctum L.	Labiatae	Sagar	14°16'N, 75°03'E, 579 m	Cough, skin disorders
Phyllanthus niruri L.	Euphorbiaceae	Shankaraghatta	13°55' N, 75°34'E, 720 m	Jaundice
Sida acuta Burm. f.	Malvaceae	Kumsi	14°4' N, 75° 24' E, 674 m	Hemorrhoids

Data analysis

The colonization rate of endophytic fungi was determined as the total number of segments yielding ≥ 1 isolate in a host sample divided by total number of segments incubated in that sample x 100. Frequency of colonization by individual taxa was calculated similarly. Significance of differences in the frequency of colonization among the host plants was determined using the Kruskal Wallis method (Gibbons 1976). Differences between winter, monsoon and summer seasons were tested by ANOVA and Similarity among the hosts and between the study sites were estimated. Shannon diversity index (H'), Shannon evenness index (J') and Simpson diversity index (1/D) were used for evaluation of fungal species richness (Zar 2004).

Results

A total of 3611 fungal isolates were recovered from 4200 leaf segments that were cut from 7 species of medicinal herbs and incubated during monsoon, winter and summer seasons. These fungal isolates belonged to teleomorphic Ascomycota (23.5%), anamorphic Ascomycota producing conidiomata (17.4%), anamorphic Ascomycota without conidiomata (46.9%), Zygomycota (1.42%) and sterile forms (10.6%) (Table 2*). Chaetomium globosum* Kunze & Schm (14%), *Aspergillus niger* Tiegh. (6.5%), *Aureobasidium pullulans* (de Bary) Arnaud. (5.7%), *Curvularia lunata* Boed. (2.7%), *Fusarium* spp. (1.30%), *Penicillium chrysogenum* Thom. (3.04%), *Pestalotiopsis* sp. (2.9%), *Trichoderma viridae* Per. Ex Fr. (1.8%), *Cladosporium cladosporioides* (Fr.) de vries. (3.54%) and sterile forms (3.02%), were isolated from more than one host plant. The total colonization rate was higher in winter than during monsoon and summer seasons (Fig 1). The colonization frequency (%) of endophytic fungi differed significantly between monsoon, winter and summer seasons (F=18.30). Maximum colonization was observed in winter followed by monsoon and summer.

The total colonization frequency ranged from 35.5% on *Lecas aspera* Spreng. to 62.3 % on *Eclipta alba* (L.) Hassk. Maximum colonization frequency was observed on *E. alba* (155%) and minimum on *A. aspera* (54.5%) during monsoon season, whilehigh colonization frequency was recorded on *E. alba* (150%) and low on *L. aspera* (66%) during the winter season. Similarly, maximum frequency of colonization was observed on *E. alba* (96%) and minimum on *E. scaber* (47.5%) during sum-

mer. *Chaetomium* was isolated as a dominant genus from *E. alba* and *Elephantopus scaber* L. Similarly *Aspergillus*, *Aureobasidium* and *Phyllosticta* were recovered as dominant endophytes from *L. aspera*, *P. niruri* and *S. acuta*, respectively. The sterile forms dominated in *A. aspera* and *O. sanctum*. The maximum fourteen endophytic fungal species were recovered from *E. alba* whereas only six species were recovered from *Achyranthes aspera L.* (Table 3). Colonization frequency (%) did not differ significantly between herbs.

Table 2: Percentage of different fungal classes encountered in some medicinal herbs

Host plants	Fungal groups (%)					Total (%)
	Asco	Asexual ascomycota		Zygo	Sterile	
		W1	W2			
Achyranthes aspera L.	-	14.8	22.3	-	28.0	65.1
Eclipta alba (L.) Hassk	59.8	25.6	46.3	-	1.8	133.6
Elephantopus scaber L.	44.5	8.6	25.8	4.1	8.0	91.1
Leucas aspera Spreng.	12.3	0.6	49.3	-	4.6	67.0
Ocimum sanctum L.	-	9.3	50.0	-	19.5	78.8
Phyllanthus niruri L.	10.1	-	69.0	4.5	2.5	86.1
*Sida acuta*Burm.f.	16.1	46.5	21.5	-	-	84.1

Notes: W1 is with conidiomata; W2 is without conidiomata.

Table 3: Percent colonization frequency, dominant genus and total number of species encountered in different medicinal herbs

Host plants	Dominant genus	Colonization frequency (%)			No. of species
		Monsoon	Winter	Summer	
Achyranthes aspera L.	Sterile form	54.5	91.5	49.5	06
Eclipta alba (L.) Hassk	*Chaetomium*	155.0	150.0	96.0	14
Elephantopus scaber L.	*Chaetomium*	104.5	121.5	47.5	12
Leucas aspera Spreng.	*Aspergillus*	70.5	66.5	64.0	08
Ocimum sanctum L.	Sterile form	86.5	102.0	48.0	09
Phyllanthus niruri L.	*Aureobasidium*	91.0	97.0	70.5	13
Sida acuta Burm.f.	*Phyllosticta*	98.5	96.5	57.5	17

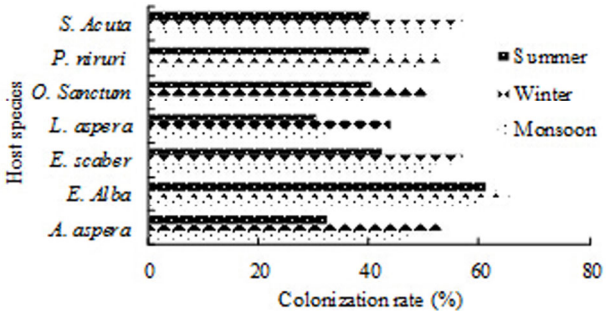

Fig. 1: Colonization rate (CR) % of endophytic fungi in different medicinal plants collected from Malnad region of Western Ghats, Karnataka

Sida acuta Burm. f. had high endophytic diversity with H¹ at1.06.Low diversity was recorded on *A. aspera* with *H'* at 0.65. Shannon Evenness was high on *O. sanctum*, with J' at 0.90 and lowest on *L. aspera* with J' at 0.78. Species abundance was high on *S. acuta* with a 1/D value of 10. Low species abundance was observed on *A. aspera* (1/D = 3.84) (Table 4). *Alternariaalternata*, *A. pullulans*, *C. globosum*, *C. lunata*, *Fusarium chlamydosporum*, *Pestalotiopsis* spp., *Sordaria fimicola* and *T. viridae isolates* recovered more during monsoon and winter, while *A. flavus*, *C. cladosporiodes*, *Fusarium* spp. recovered more during summer.

Table 4: Diversity indices of endophytic fungi in some medicinal herbs of Malnad region, Karnataka

Host plant	Shannon diversity Index	Shannon evenness (J)	Simpson diversity Index (1/D)
Achyranthus aspera L	0.65	0.84	3.84
Eclipta alba (L.) Hassk	0.90	0.78	5.26
Elephantopus scaber L.	0.93	0.86	6.66
Leucas aspera Spreng.	0.71	0.78	4.16
Ocimum sanctum L.	0.86	0.90	6.66
Phyllanthus niruri L.	0.93	0.83	5.55
Sida acuta Burm.f.	1.06	0.86	10.00

Discussion

The colonization frequency (%) of fungal endophytes in this study was within the range of the large number of host plants reported on by Suryanarayanan et al. (2003). The dominant taxa isolated in this study, including *A. pullulans*, *C. globosum*, *Pes-*

talotiospsis spp., *Penicillium* spp., and *Phyllosticta*, have repeatedly been reported as endophytes from large numbers of plants surveyed from the tropics (Rodrigues et al. 2005; Krishnamurthy et al. 2009).

Many of these endophytes colonize broad ranges of hosts and grow rapidly and competitively on non-selective, plant-based media frequently used in survey work (eg: PDA, MEA, CMA) (Lodge et al.1996; Frohlich and Hyde 1999). Many of the strains like *Aspergillus*, *Penicillium* and *Cladosporium* isolated in this study are considered as saprobic, soil or air borne fungi but these were regularly recovered from surface sterilized healthy tissues of host plants studied by Krishnamurthy et al. (2008) and Zamora et al. (2008). Maximum colonization during winter season suggests that infection levels are positively correlated with environmental factors (Bills 1996; Wilson 2000).

In many instances, leaves sampled during the wet season harbor more endophytes than those screened during the dry season (Wilson and Carroll 1994). The spores of fungi like *Colletotrichum*, *Pestalotiopsis* produce slimy conidia that are not forcibly released but dispersed by water in various ways andmightbe responsible for isolation of greater numbers of isolates during wet months (Wilson and Carroll 1994; Schulthess and Faeth1998). In winter season, humidity and moderate temperatures might enable fungal propagules to germinate successfully (Gore and Bucak 2007). The fungi such as *Aspergillus*, *Cladosporium*, and *Penicillium* have been frequently isolated during the dry season (Shankar Naik et al. 2008). Spores of these fungi can survive and even grow in a low water environment (Hyde et al. 2007).

The differences between host plants in terms of colonization by endophytes might be due to prevailing microhabitats, environment, stress, senescence of the hosts, virulence of the endophytes and host defense responses (Schulz and Boyle 2005). Inoculum volume plays an important role in determining the infection success of plant-associated fungi (Agrios 1997; Arnold 2008). The frequency of isolation of endophytic fungi also correlates with leaf fragment size, type of growth medium, culturing conditions, and surface sterilization protocols (Gamboa et al. 2002). The major challenge that remains to be addressed for clear understanding of endophyte diversity is sterile fungi that do not sporulate in media. For this reason, endophytic fungi are often grouped as morphospecies based on mycelial characteristics (Guo et al. 2003). Molecular techniques like ITS sequences and DGGE have now been effectively used for identification and classification of morphospecies of endophytic fungi (Guo et al. 2003; Jeewon and Hyde 2006). Some of the fungi isolated in this way might be sources of novel compounds and we are currently pursuing fermentation of these endophytes to obtain wide array of secondary metabolites to facilitate screening against therapeutic targets.

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